Vol. 97, No. 1, 1980
November 17, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 33-40

COCKROACH MUSCLE HEMAGGLUTININS - CANDIDATE RECOGNITION MACROMOLECULES

Jeffrey L. Denburg

Zoology Department, University of Iowa, Iowa City, Iowa 52242

Received September 24,1980

SUMMARY: Protein hemagglutinins have been detected in homogenates of each of the six coxal depressor muscles in the leg of the cockroach. The quantitative ability of some glycosaminoglycans to inhibit these hemagglutinins correlates with the muscles' innervation by identified motor neurons. There is a large decrease in activity when the muscles are denervated. Activity increases again upon reinnervation or remains low if the muscles are maintained in a denervated state.

INTRODUCTION

The neuromuscular system of the cockroach, Periplaneta americana, is an ideal one in which to test various biochemical models of intercellular recognition. Of particular interest are the interactions between two identified motor neurons (D_f and D_s) and the set of six coxal depressor muscles (CDMs) in the leg which they innervate in a fixed pattern in all individual animals (1). Muscles 178 and 179 are a homogeneous population of fibers every one of which is innervated by D_f. Muscles 177d and e are innervated only by D_s. Muscles 177d' and e' possess fibers every one of which is innervated by both D_f and D_s. When the axons of these motor neurons are disrupted, they regrow and when regeneration is completed the original innervation pattern is formed (2, 3). This suggests that a very specific intercellular recognition process takes place between identified motor neurons and muscles.

It has been suggested that intercellular recognition is mediated by macromolecules with properties similar to lectins (4). Lectins are proteins, with more than one specific carbohydrate binding site, able to form intercellular cross-links between cells resulting in their agglutination. Such macromolecules have been found in developing as well as in fully differentiated tissues (5). Red blood cells are often used to detect lectin activity

0006-291X/80/210033-08$01.00/0

because of the ease with which their agglutination can be measured and because they offer a wide range of surface carbohydrates for binding. The CDMs of the cockroach were examined for the presence of lectins in the hope that macromolecules that can cross-link red blood cells may also be responsible for specific, intercellular cross-links between identified motor neurons and muscles.

MATERIALS AND METHODS

Adult male cockroaches from the laboratory colony were used in all experiments. Individual muscles from the meso-and metathoracic legs were identified and dissected as previously described (6). Human type O, Rh negative red blood cells were obtained within 48 hours of being outdated from the local blood bank. They were trypsinized (7) and fixed with formaldehyde (8). Enzymes of highest purity available were used. Carbohydrates were obtained from Sigma and Pfanstiehl and glycosaminoglycans from Miles.

Hemmagglutinin assays were performed in microtiter V-plates in which agglutination could be easily detected (4). Serial 2-fold dilutions of muscle homogenates were made in a volume of 25 µl. The reaction was started by the addition with a syringe of 75 µl of a 1.33% suspension of red blood cells. The end point of the titration is the greatest dilution of homogenate in which agglutination occurs. In the inhibition studies, various concentrations of the inhibitor in a volume of 10 µl were added to the dilutions of the homogenate. The concentration which produced a 50% inhibition of activity (a change of one well in the end point) was recorded.

RESULTS

Hemagglutination assay conditions: In initial experiments tissue homogenates were prepared and assayed in a phosphate buffered saline (PBS) containing 150 mM NaCl, 10 mM K_2HPO_4 pH 7.3, 1 mM dithiothreitol and 1 mM EDTA. Hemagglutinating activity towards human type O cells was readily detected in homogenates of each of the CDMs as well as of the ventral nerve cord. While determining optimal assay conditions it was observed that in a dilute buffer (DB) containing 1 mM K_2HPO_4 pH 7.3, 1 mM EDTA the activity in the muscle homogenates increased to 8-16 times that in PBS while activity in neuronal homogenates could barely be detected. These non-physiological assay conditions have the advantage of enabling the measurement of only muscle hemagglutinins without any significant contribution from neuronal macromolecules from the many nerve terminals along each muscle fiber. In addition, when homogenates are prepared in DB the activity remains stable for at least 3 days at $5^{o}C$. One of the disadvantages of these assay conditions

is the increased probability of obtaining a non-specific agglutination due to increased strength of electrostatic interactions or decreased solubility of macromolecules. However, since it is likely that tissue homogenates have many different macromolecules that can agglutinate red blood cells by various mechanisms, it was decided to initially study in detail those hemagglutinating activities detected under these non-physiological conditions.

Macromolecular nature of hemagglutinins: The total inactivation of muscle hemagglutinins by incubation in boiling water for 10 minutes and by treatment with trypsin and chymotrypsin (35°C for one hour) indicates that this activity is produced by a protein maintained in a specific conformation. Treatment with deoxyribonuclease, ribonuclease, collagenase and hyaluronidase at 100 μg/ml, had no effect on hemagglutinating activity. When muscle homogenates in DB were centrifuged at 100,000g for 1 hour all the activity was recovered in the pellet. This activity could not be solubilized in the non-ionic detergents Triton X-100 and NP-40. However, extraction with 0.6M KCl or 0.1% SDS solubilized the hemagglutinins.

Inhibitors of hemagglutination: In order to determine whether the hemagglutinating activity was produced by lectins, the ability of various carbohydrates to inhibit hemagglutination was examined. Particular attention was paid towards the possibility of detecting lectins with different carbohydrate specificities in the homogenates from each of the CDMs. The results of these experiments are summarized in Table 1. Among the uncharged sugars, L-rhamnose and L-fucose were the most potent inhibitors but they inhibited the hemagglutinins from all muscles to the same extent. Galacturonic acid was the most inhibitory sugar and exhibited different affinities for the hemagglutinins from the various muscles. However, the results were variable, depending upon the source of galacturonic acid and the batch of red blood cells used. The specificity of the interaction with galacturonic acid is implied from the relatively low affinity for glucuronic acid. All of the polyanions tested, with the exception of hyaluronic acid, were potent

Table 1. Concentrations of inhibitor needed to reduce by 50% hemagglutinating activity from each coxal depressor muscle.

Inhibitor	Muscle 178, 179	177d'e'	177d,e
Uncharged sugars (concentrations in mM)			
L-Rhamnose	5-10		5-10
L-Fucose	10-20		10-20
N-Acetylgalactosamine	30		30
Deoxyglucose	50		50
Mannose	50		50
2-Methylmannoside	>100		50
Lactose	>100		20-50
D-Fucose	50-100		20-50
Deoxygalactose	100		>100
Xylose	100		100
Anionic sugars (concentrations in mM)			
N-Acetylneuraminic acid	>100		30-100
Ascorbic acid	4		4-10
Glucuronic acid	50		50
Galacturonic acid	10		5
(Pfanstiehl)	1.25-2.5	5	5-10
	2.5-5.0	1.25-2.5	1.25
(Cal Biochem)	0.63	0.31-0.63	0.16
Polyanions (concentrations in µg/ml)			
Chondroiton sulfate-A	10-20		0.5
Chondroiton sulfate-B	5-10	0.2	0.05-0.10
Chondroiton sulfate-C	1-2	0.2-0.5	0.02-0.05
Keratin sulfate	>200	20-40	5-10
Pectin	50-100	10	1
Hyaluronic acid	100		100
Heparin	0.02-0.05	0.02-0.05	0.01-0.02
Heparatin sulfate	0.1-0.2		0.05
Dextran sulfate	0.02		0.01-0.02
RNA	0.5		0.2-0.5
DNA	0.05-0.1		0.02-0.05

The following sugars did not inhibit at the highest concentration tested (100mM): glucose, galactose, trehalose, glucosamine, galactosamine, mannosamine.

inhibitors of hemagglutinating activity. However, some glycosaminoglycans (chondroiton sulfate-A,-B,-C and keratin sulfate) as well as pectin (polygalacturonic acid) inhibited by 50% the hemagglutinin from muscles 177d and e at nearly 100 times lower concentration than was needed to produce the same amount of inhibition in the hemagglutinin from 178, 179. An intermediate concentration was needed to inhibit by 50% the hemagglutinin from the doubly innervated muscles 177d', e'. The specificity of these interactions is suggested by the observation that other glycosaminoglycans (heparin and heparatin sulfate) and nucleic acids, although being potent inhibitors, could not distinguish between the hemagglutinins from the various muscles.

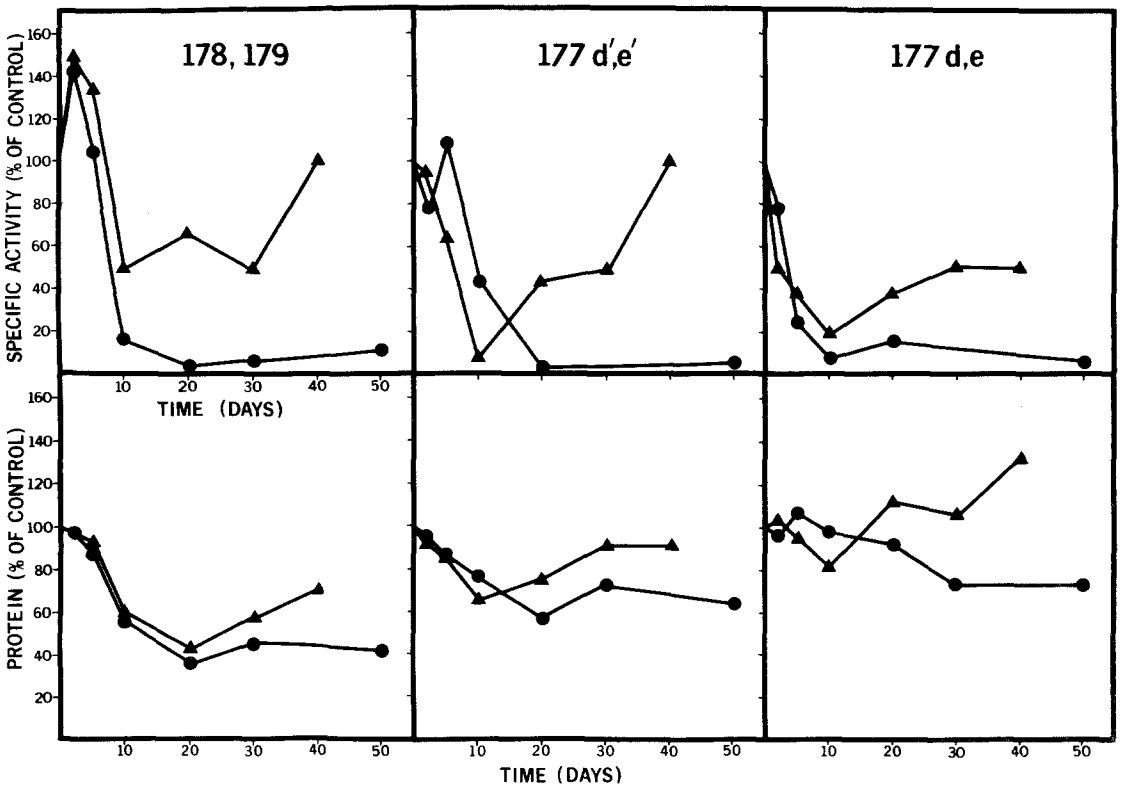

Figure 1. Changes in total protein content and specific activity of hemagglutinin at various times after denervation of the coxal depressor muscles. ●-●-● results from animals in which the nerve was cut and regeneration was prevented. ▲-▲-▲ results from animals in which the nerve was crushed and muscles became reinnervated. Innervated controls muscles are from the contralateral legs of each experimental animal.

Effect of denervation on hemagglutinating activity: The CDMs in one leg of a cockroach were denervated either by crushing the axons innervating them so that regeneration occurred rapidly or by cutting the axons in such a manner that reinnervation did not occur during the time interval of this experiment. The nerves on the other side of the animals were left intact and the CDMs in this other leg served as the innervated controls. Upon denervation the specific activity of hemagglutination changed rapidly and drastically, (Fig. 1). An initial increase in activity was followed by a great decrease. In muscles maintained in the denervated state activity remained low. In muscles being reinnervated activity rose again nearly to the original levels.

The changes in activity do not follow the atrophy of the muscles as measured by the decrease in protein content (Fig. 1). The decrease in hemagglutinating activity was not caused by the presence of an inhibitor in the denervated muscles since the mixing of homogenates of innervated control muscles and denervated ones did not cause a decrease in the initial activity. The possibility that activity requires a neuronal macromolecule present in axon terminals which degenerate upon denervation was eliminated because the addition of an homogenate of the ventral nerve cord to that of a denervated muscle did not cause an increase in activity. These results suggest that a functional innervation of the muscle regulates its levels of hemagglutinin.

DISCUSSION

The lectin hypothesis of intercellular recognition would predict that muscles 178 and 179, innervated by motor neuron D_f, would have a lectin with a carbohydrate binding site of different specificity from a lectin found in muscles 177d and e, innervated by D_s. Both lectins should be present in the doubly innervated muscles 177d' and e'. The inhibition by carbohydrates of the cockroach muscle hemagglutinins suggests that they may be lectins. However, because of the non-physiological assay conditions used it is felt that further evidence, such as specific binding to and elution from an affinity column, is required. The specificity of the inhibition by negatively charged substances is demonstrated by the observation that galacturonic acid is a more potent inhibitor than glucuronic acid, and that among the polyanions only some of the glycosaminoglycans differentially inhibit the hemagglutinins from the various muscles.

Although the cockroach muscle hemagglutinins are similar to fibronectin in that both agglutinate red blood cells (9), bind to glycosaminoglycans (10) and to nucleic acids (11), they can be distinguished by the properties of their hemagglutinating activity. Fibronectin is inhibited by EDTA and amino sugars while cockroach muscle hemagglutinins are not. A lectin that binds glycosaminoglycans was previously detected in embryonic chick muscles (12,

13). The cockroach muscle hemagglutinins differ from this macromolecule in their relative affinities for the various glycosaminoglycans.

In previous work from our laboratory one of the criteria established for a candidate recognition macromolecule was that it remain present in the denervated muscle throughout the the period of axonal regeneration (6, 14). This was based on the assumption that the specificity of the intercellular recognition was generated at the level of initial contact formation between the cells. This would eliminate the cockroach muscle hemagglutinins from consideration as recognition macromolecules since their activities decreased upon denervation. However, it has since been demonstrated that during early stages of regeneration axons grow along inappropriate paths (15) and make incorrect functional connections with muscles they do not normally innervate (3). Specificity may be generated by a process occurring after the formation of cell contacts. It is possible that the muscle hemagglutinins, present at very low levels when the initial non-specific reinnervation occurs, mediate the specific inactivation of incorrect connections or stabilization of correct ones, since their activity increases again at a time when these processes occur.

ACKNOWLEDGEMENTS

I wish to thank Ms. Judith Eastburn for skillful technical assistance. This study was supported by research grant PHS NS14295 and career development award NS 00422

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